Brown Spot Severity and Yield of Soybeans Regenerated from Calli Resistant to a Host-Specific Pathotoxin Produced by *Septoria glycines*

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ABSTRACT

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Regenerated soybean lines from the R_3 to R_7 generations were field-evaluated for their reactions to *Septoria glycines*, the brown spot pathogen, from 1991 to 1994. The regenerated lines from cvs. BSR201, Fayette, and L86P-1615 were selected from calli resistant to a host-specific pathotoxin produced by *S. glycines*. Selected resistant plants to the pathogen from the R_3 generation produced R_4 progeny that were resistant, intermediate and susceptible in their reaction to *S. glycines* in 1991. In 1992, 1993, and 1994 among the R_3 to R_7 generations, brown spot reaction was only intermediate and susceptible. F_3 families obtained from a cross between R_3 regenerants and BSR201 had low heritability (23%) for resistance to *S. glycines*. Lines in the R_5 or R_6 generation, obtained originally from plants regenerated from calli of BSR201 that had intermediate and susceptible reactions to *S. glycines* in 1992, and five commercial soybean cultivars were evaluated for brown spot resistance, maturity, and yield in 1993 and 1994. Area-under the disease progress curve (AUDPC), maturity, and yield varied among entries. The 10 lines selected with an intermediate reaction had lower AUDPC, matured later, and had higher yields than the nine susceptible lines. Three regenerated lines, 2728, 2733, and 2734, had significantly ($P = 0.05$) lower AUDPC, later maturity, and higher yields than the other regenerated lines. AUDPC was significantly ($P = 0.01$) negatively correlated to yield ($r = -0.29$), to plant height ($r = -0.74$) and number of nodes ($r = -0.39$); but positively correlated to pods having 0 seed ($r = 0.44$) and two seeds ($r = 0.49$).

Brown spot of soybeans, *Glycine max* (L.) Merr., caused by *Septoria glycines* Hemmi, is the most prevalent foliar disease of soybeans in Illinois (4,19). Brown spot causes significant yield losses to soybeans (12,18,26,28). Commercial cultivars, plant introductions, and wild *Glycine* spp. have been tested for resistance to *S. glycines* (11,13,27,29). Resistance to brown spot was detected in two accessions of *G. clandestina* Wendl. and one accession of *G. tabacina* (Labill.) Benth. (13). Some degree of resistance was detected in 19 lines of *G. max* in Russia (29). Immunity or single gene resistance in soybeans to *S. glycines* has not been reported.

Rapidly spreading leaf chlorosis is a symptom of brown spot that may be caused by a diffusible substance produced by the fungus (14). A pathotoxin from fungal culture filtrates was purified and partially characterized (21). The toxin was host-specific and caused brown spot symptoms on soybean cotyledons and leaves (21). This suggested that brown spot symptoms may be closely related to the host-specific pathotoxin produced by *S. glycines*.

Resistance to brown spot has been difficult to find by conventional screening methods. One thousand soybean plant introductions were field screened and all were classified as susceptible to *S. glycines*, with >75% leaf area diseased (11). For diseases like brown spot, for which sources of resistance have been difficult to find, somaclonal variation and in vitro selection with pathotoxic culture filtrates have been used for developing disease-resistant plants (1,6–8,22). Soybean plants with resistance to *S. glycines* were selected by means of a pathotoxic culture filtrate of *S. glycines* from cultured cells of cultivars susceptible to brown spot (22). The progenies of these regenerated plants had some level of brown spot resistance in the field (22). In this study, the progenies of those regenerated plants were tested for their brown spot reaction in the R_3 to R_7 generations. In addition, selections from the

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Table 1. Generation advancement of soybean lines obtained from calli of cvs. BSR201, Fayette, and L86P-1615, selected for resistance to a pathotoxin produced by *Septoria glycines*

	BSR201		Fayette		L86P-1615	
Year[a]	Generation	Plants or rows	Generation	Plants or rows	Generation	Plants or rows
1988	R_0[b]	5[c]	R_0	1[c]	R_0	2[c]
1989	R_1	–[d]	R_1	–	R_1	–
	R_2	832[c]				
1990	R_2	–[d]	R_2	–		–
	R_3	1,651[c]				
1991	R_3	–[d]	R_3	–	R_2	–
	R_4	1,134[c]				
1992	R_4 and R_5	1,046[c]	R_4	136[c]	R_3	936[c]
	R_5	317[e]				
	F_2 and F_3	460[c]				
1993	R_5 and R_6	100[e]	R_5	16[e]	R_4	34[e]
	F_3	1,549[c]				
	F_4	96[c]				
1994	R_6 and R_7	31[e]	R_6	14[e]	R_5	15[e]
	F_5	20[e]				

[a] Regenerated lines in 1988 to 1990 were previously advanced (22).
[b] Generation number.
[c] Number of plants evaluated following field inoculation.
[d] Unknown number of plants advanced.
[e] Number of rows evaluated following field inoculation.

R_5 and R_6 generations were evaluated for yield and brown spot resistance.

MATERIALS AND METHODS

Source of regenerants. The R_3 to R_7 lines used were advanced from R_3 lines reported to have resistance to *S. glycines*, based on reaction of calli to the toxin produced by this fungus (22). The R_3 to R_7 progeny of regenerated lines and F_2 to F_5 progeny of crosses between regenerated lines from cv. BSR201 and BSR201 (Table 1) were tested for resistance to *S. glycines* in the field.

Evaluation of resistance. From 1991 to 1994, seeds were collected from plants that had a resistant or intermediate reaction to brown spot. Seeds from each plant were sown in a single row the following year. The cvs. BSR201, Fayette, and L86P-1615 were used as checks each year. Seeds were planted in 2.4-m rows on 28 May 1991, 19 May 1992, 17 May 1993, and 20 May 1994. Plants at growth stages V2 to V6 (5) were inoculated with *S. glycines* by means of a pressurized sprayer (5.6 kg/cm^2) on 28 June, and 2 and 15 July 1991; 15 and 29 June and 10 July 1992; 22 June 1993; and 27 June and 19 July 1994. Inoculum was produced by growing *S. glycines* on potato dextrose agar (PDA) for 2 weeks at 24 ± 2°C. Cultures were macerated in a blender with tap water for 1 to 2 min and adjusted to 10^6 conidia/ml. Disease severity was rated using a modified Horsfall-Barratt scale based on visual observations of the proportion of leaf area diseased (9, 17). Disease severity and plant growth stage were evaluated weekly for 5 to 7 weeks. Reactions of individual regenerants or plant rows were classified into three categories based on percentage of leaf area diseased: resistance = 0 to 6%; intermediate = 7 to 25%; and susceptible = >25%.

Heritability test. R_3 plants from BRS201 calli that had resistance to *S. glycines* were pollinated with BSR201 in the greenhouse in 1990. Seeds of the F_1 generation (R_3F_1) were advanced to the F_2 generation (R_3F_2) in the field in 1992. Eighty-three F_3 families (R_3F_3) were randomly selected and seeds were planted along with BSR201 at the rate of 25 seeds per 75-cm row on 17 May 1993. The experiment was arranged in randomized complete block design with two replications. Inoculation method, time of inoculations, and rating scale were the same as previously described for the resistance evaluation in 1993. A total of 1,549 plants were individually evaluated for brown spot severity at growth stage R6. Severity data for individual plants in the 83 families were analyzed by analysis of variance. Genetic variability, phenotypic variability, and heritability were estimated as follows: $s^2G = [(s^2E + r\ s^2G) - s^2E]/r$, $s^2P = s^2G + s^2E$, and $h^2 = s^2G / s^2P$, respectively, where s^2G is genetic variability, s^2E is environmental variability, s^2P is phenotypic variability, and h^2 is heritability (16).

Evaluation of resistance and yield. Based on field evaluations in 1992, seeds from 10 plants that had an intermediate reaction to *S. glycines* and nine plants that were susceptible from the R_5 and R_6 generations from BSR201 calli were selected and planted along with five commercial cvs. (Asgrow A2943, BSR201, Burlison, Kenwood, and Sturdy) in 3.6-m-long two-row plots, with a distance between rows of 76 cm, in a continuous soybean cropping field on 19 May 1993. After germination, rows were trimmed to a length of 2.4 m. All plots were inoculated with a suspension of 10^6 conidia/ml concentration by means of a pressurized sprayer (5.6 kg/cm^2). Preparation of inoculum was the same as described previously. Entries were replicated three times in a randomized complete block design. The percentage of disease severity and the growth stages were visually estimated on a whole plot basis weekly from 7 July to 1 September. The area under disease progress curve (AUDPC) was calculated as: $\sum^{N} i = 1\ ((R_i + R_i + 1)/2)(t_i + 1^{-t_i})$, where t_i = day of evaluation i, R_i = percentage of disease severity at evaluation i, and i = one to eight evaluations (20). After maturity all plants in rows were harvested and dried at 37°C for 72 h to 8% moisture. Yields were adjusted to 13% moisture.

In 1994, the same 24 entries as in 1993 were planted in 4.8-m long four-row plots in a soybean-corn rotation field and in a continuous soybean cropping field. After seed germination, plant rows were trimmed to 3.6 m. A split-plot randomized complete block design was used with three replications. The inoculated and noninoculated plots were arranged as main plots and the 24 lines/cultivars were arranged as subplots. Plants were inoculated with a suspension of 10^6 conidia/ml concentration as described previously. Noninoculated plots were sprayed with five applications of benomyl (Benlate 50% WP formulation: methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate) applied at 1.1 kg/ha using 280 liters of water per ha. Disease severity was rated weekly on whole plots from 21 July to 31 August using the modified Horsfall-Barratt scale. AUDPC was calculated as described previously. At maturity, height and number of nodes on the main stem were recorded for 10 plants from each plot. Twenty plants in each plot for five regenerated lines (three best lines and two worst lines for AUDPC based on 1993 data) and BSR201 were sampled after maturity. All pods were removed and separated to determine the number of seeds per pod. The center two rows of all plots were harvested, dried, and weighed for yield as described previously. Data

Table 2. Field evaluation of brown spot in different generations of soybean lines regenerated from calli that were selected for resistance to a pathotoxin produced by *Septoria glycines*

Year	Generation and source[a]	Plants evaluated	Rows evaluated	Reaction (percentage)[b]		
				Resistant	Intermediate	Susceptible
1991	R_4P1	1,134	45	148[c] (13)	890[c] (78)	96[c] (9)
	BSR201	28	1	0	25[c] (89)	3[c] (11)
1992	R_3P2	936	36	0	82[c] (9)	854[c] (91)
	R_4P1	291	15	0	14[c] (5)	277[c] (95)
	R_4P3	136	5	0	14[c] (10)	122[c] (90)
	R_5P1	755	80	0	9[c] (1)	746[c] (99)
	F_3P1	330	18	0	11[c] (3)	319[c] (97)
	BSR201	65	2	0	0	65[c] (100)
1993	R_4P2	0	34	0	2[d] (6)	32[d] (94)
	R_5P1	0	32	0	2[d] (6)	30[d] (94)
	R_5P3	0	16	0	5[d] (31)	11[d] (69)
	R_6P1	0	68	0	3[d] (4)	65[d] (96)
	Check cultivars[e]	0	15	0	0	15[d] (100)

[a] R_3 to R_6 indicates the number of selfed generations from organogenic cultures. F_3 indicates generation of a cross between a regenerated line with BSR201. P1 indicates calli were derived from BSR201. P2 indicates calli were derived from L86P-1615. P3 indicates calli were derived from Fayette.

[b] Resistant = 1 to 6%; intermediate = 7 to 25%; susceptible = > 25% of leaf area affected.

[c] Total number of plants evaluated.

[d] Total number of rows evaluated.

[e] Cvs. BSR201, Fayette, and L86P-1615.

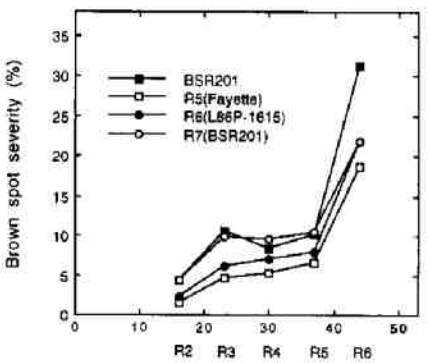

Fig. 1. Brown spot progress curves for soybean cv. BSR201 and soybean lines in the R_5 (from calli of L86P-1615), R_6 (from calli of Fayette), and R_7 (from calli of BSR201) generations after inoculation with *Septoria glycines* in the field in 1994.

Table 3. Field evaluation of brown spot on soybean cv. BSR201 regenerated lines that were selected one generation earlier based on their reaction to *Septoria glycines*[a]

Reaction[b]	Generation[c]	No. of rows	Reaction (percentage)	
			Intermediate	Susceptible
Intermediate	R_5	293	42[d] (14)	251 (86)
Intermediate	F_4	51	3 (4)	50 (96)
Intermediate	F_5	4	4 (100)	0 (0)
Susceptible	R_5	24	0 (0)	24 (100)
Susceptible	F_4	45	0 (0)	45 (100)
Susceptible	F_5	6	0 (0)	6 (100)
Susceptible	BSR201	14	0 (0)	14 (100)

[a] Disease severity based on ratings at growth stages R_4 to R_5 from 1992 to 1994.
[b] Intermediate = 7 to 25% and susceptible = >25% leaf area diseased.
[c] R_5 is the fifth-selfed generation. F_4 and F_5 are the fourth- and fifth-selfed generations from R_3 and R_2 regenerants, respectively, crossed to BSR201. R_5, F_4, and F_5 generations were evaluated in 1992, 1993, and 1994, respectively.
[d] Total number of rows evaluated.

Table 4. Area under disease progress curve (AUDPC) and relative rank, maturity date, and yield and relative rank for 19 soybean lines regenerated from organogenic cultures of cv. BSR201 and five commercial cultivars inoculated with *Septoria glycines* in the field in 1993

Lines/cultivars	AUDPC		Maturity date	Yield	
	Value	Rank		Kg/ha	Rank
Intermediate[a]					
2322	1599	5	Sept. 14	3,172	7
2335	1630	7	Sept. 13	2,972	17
2354	1605	6	Sept. 14	3,114	12
2387	1681	12	Sept. 13	3,015	14
2681	1649	8	Sept. 14	2,991	15
2700	1652	9	Sept. 14	3,156	9
2709	1574	4	Sept. 14	3,123	11
2728	1292	3	Sept. 19	3,185	6
2733	1290	2	Sept. 18	3,511	2
2734	1242	1	Sept. 20	3,113	13
Susceptible[b]					
2281	1735	17	Sept. 12	3,159	8
2284	1763	19	Sept. 11	2,811	23
2325	1811	24	Sept. 11	2,836	22
2330	1786	22	Sept. 10	2,954	18
2442	1713	15	Sept. 10	2,793	24
2447	1725	16	Sept. 11	2,977	16
2455	1796	23	Sept. 10	2,858	21
2692	1696	14	Sept. 12	2,932	19
2699	1658	10	Sept. 13	3,134	10
Check cultivars					
ASG2943	1666	11	Sept. 18	3,412	4
BSR201	1739	18	Sept. 14	2,928	20
Burlison	1695	13	Sept. 17	3,450	3
Kenwood	1778	21	Sept. 14	3,672	1
Sturdy	1774	20	Sept. 10	3,203	5
FLSD[c]	90			220	
Means					
Intermediate/10 lines	1521		Sept. 15	3,135	
Susceptible/9 lines	1742		Sept. 11	2,939	
Check cultivars	1730		Sept. 15	3,333	
Three lowest AUDPC lines[d]	1274		Sept. 19	3,268	
Comparisons					
Intermediate vs. susceptible	0.01[e]		0.01	0.01	
Intermediate vs. checks	0.01		NS[f]	0.01	
Susceptible vs. checks	NS[f]		0.01	0.01	
Three lowest AUDPC lines vs. BSR201	0.01		0.01	0.01	

[a] Intermediate = 10 lines with intermediate reaction from progenies of R_5 and R_6 generations.
[b] Susceptible = nine lines with susceptible reaction from progenies of R_5 and R_6 generations.
[c] Fisher's protected least significant difference ($P = 0.05$).
[d] Three lowest AUDPC lines = 2728, 2733, and 2734.
[e] Means differ significantly at $P = 0.01$.
[f] Not significant.

were analyzed by analysis of variance. Entries were divided into two groups, inoculated and noninoculated plots, and the means were separated by Fisher's protected least significant difference and group mean comparison.

RESULTS

Evaluation of resistance. In 1991, 148 of 1,134 R_4 generation plants were classified as resistant, 890 as intermediate, and 96 along with BSR201 as susceptible (Table 2). In 1992, 1 to 10% of the regenerants had an intermediate reaction, and all the rest were susceptible, as was BSR201 (Table 2). In 1993, no plants were rated resistant (Table 2). Only 4 to 31% of the 150 rows in the different generations had an intermediate reaction, and all the rest including the check cultivars were susceptible (Table 2). The disease classification of regenerants varied based on the source of parental calli. For example, regenerants in the R_4 and R_5 generations from calli of Fayette and L86P-1615 had more plants with an intermediate reaction (10 and 9%, respectively) than those from BSR201 in the R_5 and R_6 generations in 1992 and 1993 (Table 2).

BSR201 and three lines selected based on an intermediate reaction that were derived from calli of cvs. BSR201, Fayette, and L86P-1615 had slow disease development in 1994 until 38 days after inoculation, at which time brown spot greatly increased (after growth stage R5) (Fig. 1). Disease progress curves for BSR201 and the three lines were similar until growth stage R5. After growth stage R5 all selected lines had significantly ($P = 0.01$) lower disease severity than BSR201 (Fig. 1).

The R_5, F_4, and F_5 lines selected for susceptibility in the previous year were susceptible the next year. R_5 and F_4 lines selected for their intermediate reaction in the previous year varied from intermediate to susceptible, while F_5 lines from the intermediate reaction in the previous year were intermediate the next year (Table 3).

Heritability test. The mean square variance for 83 R_3F_3 families was 27 and the error mean square variance was 17. The genetic variance was 5, environmental variance was 17, and phenotypic variance was 22. Heritability of disease resistance in R_2F_3 families was 23% of the total genetic variance.

Evaluation of resistance and yield. In 1993, AUDPC varied among the entries from 1,242 to 1,811 (Table 4). Mean AUDPC of the 10 intermediate lines was significantly ($P = 0.01$) less than the mean AUDPC of the nine susceptible lines and the five check cultivars. Maturity also varied among the entries from 10 to 20 September (Table 4). The intermediate lines matured later than the susceptible lines, but had a maturity similar to that of the five check cultivars. Yield also varied

among the entries from 2,793 to 3,672 kg/ha (Table 4). Average yields of the 10 intermediate lines (3,135 kg/ha) was significantly ($P = 0.01$) higher than the average yield of 2,939 kg/ha for the nine susceptible lines (Table 4). Yields of three lowest AUDPC lines were significantly ($P = 0.01$) higher than that of BSR201.

When benomyl was used to reduce brown spot severity, maturity and yield varied among entries in 1994 (Table 5). The AUDPC of the 10 intermediate lines was similar to that of the nine susceptible lines and the five check cultivars. Days to maturity of the 10 intermediate lines were delayed by 6 days compared with the susceptible lines and BSR201. The 10 intermediate lines had a yield similar to that of the nine susceptible lines and the five check cultivars. BSR201 ranked 14th for yield and had a yield similar to that of the three lowest AUDPC lines.

In the inoculated trial in 1994, mean AUDPC of the 10 intermediate lines was significantly ($P = 0.01$) lower than mean AUDPC of the nine susceptible lines and mean AUDPC of the five check cultivars (Table 6). BSR201 ranked 18th for AUDPC among 24 entries. The 10 intermediate lines matured later than the nine susceptible lines and BSR201. Three lines (2728, 2733, and 2734) had significantly ($P = 0.01$) lowest AUDPC compared with the other intermediate and susceptible lines, and check cultivars. Yields of the 10 intermediate lines were significantly ($P = 0.01$) higher than those of the nine susceptible lines and BSR201, but were similar to the five check cultivars.

When comparing AUDPC between the inoculated and fungicide-protected plots, the AUDPC in the inoculated plots for the 10 intermediate lines was less than that of the nine susceptible lines and the five check cultivars (Table 7). Days to maturity increased for plants in fungicide-protected plots compared with inoculated plots with BSR201 having the greatest difference. The yield loss of the 10 intermediate lines was lower than that of the nine susceptible lines and the five check cultivars. BSR201 had 16% less yield in the inoculated trial than in the protected trial.

AUDPC was significantly ($P = 0.01$) negatively correlated to yield ($r = -0.29$), to plant height ($r = -0.74$) and number of nodes ($r = -0.39$); but positively correlated to pods having 0 seed ($r = 0.44$) and two seeds ($r = 0.49$).

DISCUSSION

Pathotoxins have been used for the selection of in vitro resistance for alfalfa, corn, potato, soybean, and tobacco (1,6–8, 22,24). In vitro selection and regeneration of resistant soybean plants to *S. glycines* in the R_2 and R_3 generations were reported under field screening (22). Regenerated plants when inoculated with *S. glycines* at growth stage V3 had no brown spot until growth stage R6. All resistant plants were shorter, and varied in maturity and sterility (22). Selection of both resistant and susceptible regenerated plants produced progeny that varied in resistance to *S. glycines* (22). In our study, the regenerants from the R_3 to R_7 generations differed from the R_2 and R_3 generations in resistance to brown spot. Brown spot resistance was not observed in the R_3 to R_7 generations from 1992 to 1994 although a few plants were classified as resistant in 1991. The growing season in 1991 was drier than that of other years, which reduced the severity of brown spot. Lines susceptible to brown spot produced susceptible progeny the next year whereas lines intermediate to brown spot produced progenies that varied from intermediate to susceptible. Variations for other characteristics, including plant height, maturity, and sterility, were detected only from calli of immature embryo selections in a limited number of families. The level of brown spot resistance reported in the R_2 and R_3 generations derived from immature organogenesis (22) was not maintained in advanced generations.

In order for in vitro selection to be successful, traits should be heritable. This has not, however, always been true, since resistant variants expressed in culture were not always resistant in regenerated plants (3, 25). In addition, other traits have been shown to be lost upon regeneration (2,15). In our study, most of the selected lines in the R_7 generation were susceptible to *S. glycines*. Also a few of the selected lines from calli of immature seeds of BSR201 were not stable for resistance to *S. glycines*, or for maturity and sterility. Lines regenerated from different calli may differ

Table 5. Area under disease progress curve (AUDPC) and relative rank, days to maturity, and yield and relative rank of 19 soybean lines regenerated from organogenic cultures of cv. BSR201 and five commercial cultivars following protection with benomyl in the field in 1994

	AUDPC			Yield	
Lines or comparison	**Value**	**Rank**	**Maturity date**	**Kg/ha**	**Rank**
Intermediate[a]					
2322	27	5	Sept. 12	2,544	23
2335	35	18	Sept. 12	2,993	10
2354	37	19	Sept. 14	3,165	4
2387	38	21	Sept. 13	2,906	13
2681	33	10	Sept. 16	3,108	7
2700	26	2	Sept. 13	2,815	17
2709	39	22	Sept. 15	3,119	6
2728	24	1	Sept. 16	2,695	21
2733	34	13	Sept. 19	3,163	5
2734	33	11	Sept. 19	3,460	1
Susceptible[b]					
2281	26	3	Sept. 11	2,510	24
2284	35	16	Sept. 11	2,947	12
2325	35	17	Sept. 12	2,818	16
2330	34	12	Sept. 14	2,876	15
2442	28	6	Sept. 8	2,559	22
2447	38	20	Sept. 10	2,789	18
2455	43	24	Sept. 9	2,742	20
2692	35	15	Sept. 13	3,073	8
2699	34	14	Sept. 13	3,003	9
Check cultivars					
ASG2943	31	8	Sept. 17	3,377	2
BSR201	40	23	Sept. 13	2,880	14
Burlison	29	7	Sept. 14	2,773	19
Kenwood	27	4	Sept. 12	3,359	3
Sturdy	31	9	Sept. 9	2,984	11
FLSD[c]	NS[d]			372	
Means					
Intermediate/10 lines	32		Sept. 15	2997	
Susceptible/9lines	34		Sept. 11	2813	
Check cultivars	32		Sept. 13	3075	
Three lowest AUDPC lines[e]	30		Sept. 18	3106	
Comparisons					
Intermediate vs. susceptible	NS		0.01	NS	
Intermediate vs. check cultivars	NS		0.01	NS	
Susceptible vs. check cultivars	NS		0.05	0.05	
Three lowest AUDPC lines vs. BSR201	0.05[f]		0.01	NS	

[a] Intermediate = 10 lines with intermediate reaction from progenies of R_5 and R_6 generations.
[b] Susceptible = nine lines with susceptible reaction from progenies of R_5 and R_6 generations.
[c] Fisher's protected least significant difference ($P = 0.05$).
[d] Not significant.
[e] Three lowest AUDPC lines = 2728, 2733, and 2734.
[f] Means differ significantly at $P = 0.05$.